Deleterious effects of endogenous and exogenous testosterone on mesenchymal stem cell VEGF production

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Submitted 14 December 2007; accepted in final form 11 March 2008

Ray R, Herring CM, Markel TA, Crisostomo PR, Wang M, Weil B, Lahm T, Meldrum DR. Deleterious effects of endogenous and exogenous testosterone on mesenchymal stem cell VEGF production. Am J Physiol Regul Integr Comp Physiol 294: R1498–R1503, 2008. First published March 12, 2008; doi:10.1152/ajpregu.00897.2007.—Modulating the paracrine effects of bone marrow mesenchymal stem cells (BMSCs) may be important for the treatment of ischemic myocardial tissue. In this regard, endogenous estrogen may enhance BMSC vascular endothelial growth factor (VEGF) production. However, little information exists regarding the effect of testosterone on stem cell function. We hypothesized that 1) endogenous or exogenous estrogen will enhance stem cell production of VEGF and 2) endogenous or exogenous testosterone will inhibit BMSC VEGF production. BMSCs were collected from adult male, female, castrated male, and ovariectomized female rats. One hundred thousand cells were incubated with testosterone (1, 10, or 100 nM) or estrogen (0.15, 1.5, or 15 nM) for 48 h. Cell supernatants were collected, and VEGF was measured by ELISA. BMSCs harvested from castrated males, normal females, and ovariectomized females produced more VEGF compared with normal males. Castration was associated with the highest level (1.015 ± 98.26 pg/ml) of VEGF production by BMSCs, which was significantly more than that produced by BMSCs harvested from normal male and normal female animals. Exogenous testosterone significantly reduced VEGF production in BMSCs harvested from ovariectomized females in a dose-dependent manner. Exogenous estrogen did not alter BMSC VEGF production. These findings suggest that testosterone may work on BMSCs to decrease protective growth factor production and that effective removal of testosterone’s deleterious effects via castration may prove to be beneficial in terms of protective factor production. By manipulating the mechanisms that BMSCs use to produce growth factors, we may be able to engineer stem cells to produce maximum growth factors during therapeutic use.

sex; sex hormones; estrogen; castration; ovariectomy; androgens

Bone marrow mesenchymal stem cells (BMSCs) are a novel therapeutic approach for the treatment of myocardial ischemia (10, 34, 35). The beneficial effects of stem cells may be partially mediated through their paracrine properties (5, 33, 46). Sex dimorphisms have been noted within mesenchymal stem cell function, because female BMSCs express higher levels of protective growth factors in response to injury compared with male BMSCs (4). Differences in growth factor expression have partially been attributed to these sex dimorphisms, and in this regard, endogenous estrogens likely play a role in stem cell paracrine effects (17).

Estrogen and its receptors appear to play important roles in stem cell function (50). Endothelial progenitor cells (EPCs) cultured from human donors showed expression of estrogen receptor (ER)-α and increased proliferation when exposed to 17β-estradiol in a dose-dependent manner (11). Furthermore, the migration of EPCs into ischemic border zones was impaired in ER-α knockout mice. Downregulation of vascular endothelial growth factor (VEGF) production was also noted in EPCs from ER-α knockouts both in vivo and in vitro (17).

Despite increasing evidence to suggest that estrogen plays a beneficial role in stem cell function, little evidence exists as to the role that androgens may play on BMSC function and growth factor production. Studies on human subjects with low testosterone levels showed a significant reduction in both circulating and endothelial progenitor cells. In these patients, testosterone replacement therapy induced a significant increase in these progenitors with respect to baseline. Furthermore, castration has been shown to decrease thrombocytopenia, while testosterone administration restored platelet production (43).

Most reports of beneficial testosterone effects (12), multiple studies exist to suggest that testosterone may be deleterious to the function of certain cells, including hematopoietic precursor cells. Ablation of testosterone activity by castration was shown to increase circulating B cell progenitors (9). Other studies reported minimal survival-enhancing or growth-stimulatory effects of androgens on hematopoietic progenitor cells (26). Furthermore, studies examining androgen receptors on embryonic stem cells have suggested that androgen blockade may enhance stem cell proliferation and increase Akt production in these cells (3). Other studies determined that testosterone sharply reduced the percentages of preadipocytes in the inguinal fat depot, but it had no effect on the retroperitoneal preadipocyte pool (21), suggesting that the effects of androgens may be site- and/or tissue specific (15).

In the attempt to engineer the optimal stem cell designed to provide maximum growth factor production during ischemia, it becomes essential to elucidate the mechanisms by which stem cells produce these growth factors. In this regard, understanding the role that endogenous and exogenous sex hormones play in BMSC VEGF production becomes essential. Therefore, we hypothesized that 1) endogenous or exogenous estrogen will enhance production of VEGF by stem cells and 2) exogenous or endogenous testosterone will decrease stem cell VEGF production.

METHODS

Animals. Normal adult male, female, ovariectomized female, and castrated male Sprague-Dawley rats were fed a standard diet and

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acclimated in a quiet quarantine room for 1 wk before the experiments. Ovariectomized and castrated animals were killed 6 wk after their surgical interventions. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1985).

Preparation of mouse bone marrow stem cells. A single-step purification method using adhesion to cell culture plastic was employed. Rat bone marrow stromal cells were collected from bilateral femurs and tibias by removing the epiphyses and flushing the shafts.
with complete medium (Iscove’s modified Dulbecco’s medium and 10% fetal bovine serum; GIBCO Invitrogen, Carlsbad, CA), using a syringe with a 23-gauge needle. Cells were disaggregated by vigorous pipetting several times and were passed through a 30-μm nylon mesh to remove remaining clumps of tissue. Cells were washed by adding complete medium, centrifuging for 5 min at 300 rpm at 24°C, and removing the supernatant. The cell pellet was then resuspended and cultured in 75-cm² culture flasks with complete medium at 37°C. BMSCs preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in the suspension were discarded. Fresh complete medium was added and replaced every 3 or 4 days thereafter. BMSC cultures were maintained at 37°C in 5% CO₂ in air. At 80% confluence, cells were collected by the addition of a solution of 0.25% trypsin-EDTA (GIBCO Invitrogen) and divided into seven 75-cm² flasks. Cells were used for experimentation during passages 3–7.

Experimental groups. BMSCs were divided into male, female, castrated male, and ovariectomized female groups. While in culture flasks, BMSCs were exposed to 1) no stimulus, 2) estrogen (0.15, 1.5, or 15 nM), or 3) testosterone (1, 10, or 100 nM) for 48 h before experimentation. The lowest values of the selected doses represent the average physiological peak values for the animals (22, 41). After 48 h, BMSCs were plated in 12-well plates at a concentration of 100,000 cells·well⁻¹·ml⁻¹ (triplicates per well) in nonhormone medium for an additional 24 h. To measure the level of growth factors released from the BMSCs in a sustained fashion (genomic effects of the hormones), we collected the supernatants at 24 h after the hormone treatment. At least six independent experiments were carried out for each group of stem cells (n = 6) to confirm the accuracy of results.

Enzyme-linked immunosorbent assay. VEGF production by the BMSCs was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (R&D Systems, Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions. All samples and standards were measured in duplicate.

Presentation of data and statistical analysis. All reported values are means ± SE. Data were compared by Student’s t-test. A two-tailed probability value of <0.05 was considered statistically significant. We selected the data of experimental groups treated with the 0.15 nM dose of estrogen because this is the physiological dose of estrogen for normal proestrous rats and there was no significant difference in the results with other doses of estrogen. For testosterone treatment groups, we selected the 100 nM dose of testosterone because we found a significant difference among experimental groups with this dose only.

RESULTS

Endogenous testosterone is detrimental to BMSC VEGF production. BMSCs harvested from normal male rats produced VEGF under normal culture conditions (504.3 ± 41.19 pg/ml). Male castration increased BMSC VEGF production above that seen in normal males (1,018 ± 98.26 pg/ml). Furthermore, stem cells from both normal females and ovariectomized females produced higher levels of VEGF compared with males under normal culture conditions (females: 642.9 ± 43.47 pg/ml; ovariectomized females: 850.5 ± 82.18 pg/ml) (Fig. 1). These findings
suggest an inhibitory effect of endogenous testosterone on BMSC VEGF production, an abrupt absence of which caused a surge of rebound overproduction in castrated animals.

**Effect of exogenous testosterone on BMSC VEGF production.** The application of increasing doses of exogenous testosterone to BMSCs had no effect on normal male, castrated male, or normal female BMSC VEGF production. However, exogenous testosterone significantly reduced VEGF production in ovariectomized female BMSCs in a dose-dependent manner (control: 850.5 ± 82.18 pg/ml; 1 nM: 775 ± 52.29 pg/ml; 10 nM: 623.8 ± 68.11 pg/ml; 100 nM: 404.6 ± 50.06 pg/ml) (Fig. 2). The inhibitory effects of testosterone on BMSC VEGF production were more obvious in ovariectomized animals deficient in endogenous estrogen. When the different groups of BMSCs treated with exogenous testosterone (100 nM) were compared directly, castrated male BMSCs continued to exhibit significantly higher levels of VEGF (Fig. 3).

**Effect of exogenous estrogen on BMSC VEGF production.** Increasing doses of exogenous estrogen had no effect on normal male, castrated male, normal female, or ovariectomized female BMSC VEGF production (Fig. 4).

When the different groups of BMSCs treated with exogenous estrogen (physiological dose = 0.15 nM) were directly compared, castrated male BMSCs continued to exhibit significantly higher levels of VEGF (1.105 ± 119.5 pg/ml) compared with normal male BMSCs (527 ± 91.76 pg/ml). In response to exogenous estrogen, ovariectomized female BMSCs produced significantly more VEGF (852.9 ± 100.4 pg/ml) compared with normal female BMSCs (564.2 ± 42.12 pg/ml), describing the stimulatory effect of exogenous estrogen (Fig. 5).

**DISCUSSION**

This study was designed to examine the effects of endogenous and exogenous sex hormones on mesenchymal stem cell VEGF production. Here we confirmed that female mesenchymal stem cells produce more VEGF compared with male cells. Furthermore, we showed that 1) removal of testosterone via male castration increased VEGF production beyond that of normal males, normal females, or ovariectomized females; 2) exogenous testosterone only decreased VEGF production in stem cells harvested from ovariectomized females; and 3) exogenous estrogen had no effect on BMSC VEGF production.

In response to ischemia, such as during myocardial infarction, BMSCs and EPCs migrate to the sites of injury and play a protective role to native cells through a variety of mechanisms (1, 13, 17, 45). These include differentiation of stem cells into mature end organ cells as well as increased neovascularization of native tissue (23, 27). However, the immediate effects of stem cell protection are believed to be mediated through the paracrine release of protective growth factors such as VEGF (50).

VEGF has been shown to chronically inhibit leukocyte/epithelial cell adherence and the effects of chronic inflammation (38). This may be due to the recruitment of native stem cells, because VEGF gene transfer has been shown to mobilize EPCs in human subjects (24, 25). In addition, various tissue studies have demonstrated that VEGF promotes angiogenesis during acute inflammation and ischemia, which may work to improve functional recovery after injury (16, 31, 47, 53, 56). BMSC activation is often associated with increased VEGF production, and this may protect ischemic myocardium via upregulation of hypoxia-inducible factor-1 (7, 53). Female BMSCs also produce more VEGF than males, and recent studies suggest that administration of female BMSCs before myocardial ischemia provides superior protection, which may be attributed to the protective effects of endogenous estrogen (8, 30, 42, 49, 52).

Testosterone has previously been shown to play a detrimental role in the function of cells from many different tissues. Many studies examining the effects of testosterone on the heart suggest that testosterone may exhibit its deleterious effects by altering lipoprotein metabolism or by initiating defective cardiac remodeling (18, 29, 36, 37, 54). Myocardial ischemia-reperfusion and trauma-hemorrhage models have demonstrated improved postischemic functional recovery in castrated mice and in mice treated with testosterone receptor blockers compared with normal mice (40, 51). Other studies examining the effects of castration, ovariectomy, and hormone manipulation on myocardial infarction suggest that testosterone administration may play a key role in the induction of inflammatory cell infiltration at the infarct border zone and may serve to increase the infarct expansion index rate of myocardial rupture (2).

Therefore, on the basis of these previous studies, it makes intuitive sense that male castration would increase BMSC VEGF production because of the lack of testosterone signaling within these cells. Furthermore, by ovariectomizing the female and then exposing these BMSCs to exogenous testosterone, we noted a significant decrease in VEGF production, effectively transforming the female into a male in terms of VEGF production. This would suggest that the presence of either endogenous or exogenous testosterone is detrimental to stem cell VEGF production. Other metabolites of testosterone, such as 5-dihydrotestosterone, may be even more detrimental to stem cell VEGF production. However, it is not known whether stem cells inherently possess 5α-reductase to convert testosterone to...
this more active form. Therefore, we elected to focus solely on testosterone for the purposes of this study.

Only the ovarioctomized female cells, and not the other cell lines, exhibited decreased VEGF production after exogenous testosterone application. It is highly conceivable that the normal male and castrated male cells had downregulated androgen receptors due to previous chronic testosterone exposure and therefore did not respond to the doses of testosterone that we used. In addition, the presence of estrogen (4-fold higher in females compared with males; Ref. 22) may have balanced the deleterious effects of testosterone (30-fold lower in females compared with males; Ref. 22) in the normal female, thereby effectively abating the exogenous testosterone signaling. Further studies examining the effects of more superphysiological doses of testosterone are likely required to elucidate these mechanisms.

The presence of estrogen has been shown to provide beneficial effects to many tissues, including the production of vital growth factors (20, 39, 44). Females continuously perform better after injury, and this has previously been attributed to endogenous estrogen (19, 32). In addition, these same sex dimorphisms have been shown to exist in BMSC function after noxious stimulation. Therefore, the application of exogenous estrogen to stem cells might reasonably increase the production of protective growth factors, such as VEGF. Unexpectedly, though, application of exogenous estrogen in this study did not yield further elevations in stem cell VEGF production. This may indicate that different signaling cascades are utilized for endogenous and exogenous estrogen, but it more likely suggests that supraphysiological levels of exogenous estrogen are required for increased VEGF production to be observed.

Perspectives and Significance

Morbidity and mortality rates from coronary artery disease are significantly higher in males compared with age-adjusted females (54). Testosterone mediates its proatherogenic functions through altered lipoprotein metabolism, increased lipid accumulation in macrophages, and enhanced platelet aggregation (28, 48, 55). In animal models of cardiac ischemia, testosterone enhanced pathologic remodeling through an increase in afterload to the heart (14). Moreover, we previously demonstrated that acute testosterone infusion depressed myocardial functional recovery and enhanced apoptotic signaling. Conversely, we saw that testosterone depletion by castration or androgen receptor blockade by flutamide enhanced myocardial recovery following ischemia-reperfusion injury (6, 51). However, the role of testosterone in various progenitor cell and stem cell functions is understudied. Therefore, we designed our present study to reveal the effects of testosterone on BMSC function. The findings of this study indicate that endogenous and exogenous testosterone is detrimental to BMSC VEGF production and suggest that the improved outcomes seen in females after certain forms of injury might not necessarily reflect the protective effects of estrogen. Rather, the absence of the deleterious effects of testosterone in females may be the underlying mechanism to explain the observed outcomes. In this regard, the effective removal of testosterone’s deleterious effects via antiandrogen therapies may yield a significant impact for the stem cell therapy of myocardial ischemia. Enhanced growth factor production by androgen ablation may increase the potency of the BMSCs manifold for therapeutic uses. Further understanding of sex hormone signaling mechanisms within BMSCs may allow for the ex vivo priming of these cells in order to harvest maximum beneficial growth factor production.

GRANTS

This work was supported in part by National Institutes of Health Grants ROI-GM-070628, R01-HL-085595, K99/R00-HL-087607-01, and F32-HL-085982, an American Heart Association (AHA) Grant-in-aid, and AHA Postdoctoral Fellowship 072566Z.

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